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Title: *Protein Logic Gates*

DECLARATION UNDER 37CFR1.132

I, Professor Henry Bourne declare and state as follows:

1. I am a Professor in the Department of Cellular and Molecular Pharmacology at the University of California, San Francisco. I received my MD degree from Johns Hopkins and took my postdoctoral training at the National Institutes of Health and UCSF. I became a faculty member at UCSF in the Department of Medicine in 1971, and served as chair of the Department of Pharmacology from 1984 to 1992. I have authored numerous scientific papers in the field of protein engineering. I am familiar with this patent application and the related ongoing work in the Lim laboratory.

2. The claimed invention is an autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain. The fusion proteins link protein input and output domains that are normally not related to provide protein signaling switches analogous to logic gates with diverse and novel input/output properties.

The selected input and output domains are discretionary to the user according to intended use, and essentially any output domain providing a desired activity or binding affinity may be employed, so long as output activity can be regulated by ligand-dependent interaction of the input domains (e.g. Specification, p. 6, lines 13-15). Similarly the selection of input domains is user discretionary, so long as the selected domains interact to provide the requisite ligand-dependent gating of the output domain (e.g. Specification, p.7, lines 18-19).

For example, output domain functional compatibility with the fusion proteins is readily confirmed in routine activity screens (e.g. Specification, p.6, lines 16-17). A wide variety of output activities may be obtained, depending on the ultimate user application, including catalytic, label-generative, metabolic, apoptotic, and specific-binding output activities (Specification, p.6, lines 17-19). Table 1 lists exemplary output domains shown to have regulatable output activities, including well-studied kinase, phosphatase and protease domains (Specification, p.6, line 25 – p.7, line 7).

Similarly, input domain functional compatibility (demonstrating gating behavior) with the fusion proteins is readily confirmed in routine activity screens. A wide variety of interacting input domains may be used, depending on the ultimate user application, including peptide hormones and cognate receptor ligand binding domains (LBD), immune receptors and cognate antigenic peptides, src-homology domains and cognate peptide ligands, and various catalytic input domains, including modular proteases and both cleavable and non-cleavable pseudosubstrate peptides, modular kinases and peptide substrates, modular phosphatases and phospho-peptide substrates, etc. The input domain interaction can be provided by homo- or hetero-dimerization, by specific pair binding, by higher order complex formation, by enzyme-substrate catalysis (e.g. phosphorylation, glycosylation, prenylation, acylation, lipid modification, etc.). Specification, p.7, lines 18-30.

Preferred input domains comprise native, modular interacting domains which mediate binding of naturally interacting proteins, or natural, modular receptors or enzymes and their cognate ligands and substrates. A wide variety of such modular interacting components has been identified, categorized and subject to grafting. In addition, suitable input domains may be derived from vast public databases of known interacting proteins, including Database of Interacting Proteins (DIP), Database of Ligand-Receptor Proteins, Java-based DIP, and LiveDIP; see, e.g. Xenarios, et al. (2002) NAR 30:303-5; Xenarios, et al.(2001) NAR 29:239-41; Xenarios et al., (2000) NAR 28:289-91; Deane et al.(2002) Mol Cell Prot 1:349-356; Graeber et al. (2001) Nat.Genet. 29:295-300; Marcotte et al. (2001) Bioinformatics 17:359-63; Salwinski et al. (2003) Mol Cell Proteomics. 2002 May;1(5):349-56; Xenarios et al. (2001) Curr Opin Biotechnol 12:334-339. In addition, many protein interaction domains can be mutated to provide alternative specificity binding partners. For example, mutation of a threonine residue of the Src SH2 domain to tryptophan converts ligand-binding specificity from the Src-like pTyr-Glu-Glu-Ile (SEQ ID NO:1), to the signature Grb2 binding motif pTyr-X-Asn (Kimber et al. Molecular Cell 2000. 5, 1043-1049). Table 2 lists exemplary input domain binding pairs shown to have external ligand regulatable binding. Specification, p. 8, line 5 – p.18, line 22).

To promote their interactions, one or more of the input domains may be coupled to the fusion protein through a linker or spacer peptide. Linker peptides are widely used in fusion proteins. Linker sequence and length are user-discretionary, though the linkers should not interfere with the output domain when the switch is in the active state (e.g. de-repressed), which is readily confirmed empirically. Preferred linkers often provide structural flexibility and mobility to the input domain. Exemplary use of linker peptides is provided in the disclosed examples of exemplary fusion proteins. Specification, p.7, lines 31 – p.8, line 4.

The claimed protein switches are readily designed or screened such that external ligand activation up-regulates, down-regulates, or otherwise alters output activity. For example, activation can increase, decrease or alter label expression, binding or substrate affinity or specificity, etc. In particular embodiments, the output domain is constitutively active or functional, and in the absence of the ligand, the input domains interact to inhibit the output domain. Where the selected output domain also comprises a suitable input or interaction domain, this endogenous interaction domain may be exploited to create novel allostery in conjunction with a heterologous input or interaction domain. Typically, such endogenous input domains are positioned on the output domain so as to not interfere with the output activity, e.g. the output activity when the fusion protein is de-repressed with ligand. Specification, p.7, lines 8-17.

A wide variety of external ligands may be used to activate the switches by interacting with one or more of the input domains. The external ligands may activate reversibly, such as by reversible competitive or allosteric interaction with one or more of the input domains, or may

activate irreversibly, such as through covalent modification. For example, in the case of an SH3 input domain, proline rich peptides can be used as both a second, integral input domain, and as an external competitive ligand. Alternatively, the external ligand can comprise a kinase activity which phosphorylates (covalently modifying) the SH3 domain proximate to the proline-rich binding site, and thereby disrupts interaction of the input domains. Specification, p.18, line 24 – p.19, line 2.

In particular embodiments, the fusion proteins comprise two input domains, both heterologous to the output domain, and which form a specific binding pair. In these embodiments, the input domains may also be referred to as receptor-ligand pairs, wherein this internal ligand is one of the input domains, as opposed to the actuating, external ligand which competitively or allosterically disrupts pair-specific binding of the input domains. This input domain binding pair motif may be expanded with additional input domains to provide any desired form of cooperative or antagonistic regulation. For example, the fusion protein may comprise two or more specific binding pairs of input domains which provide higher-order cooperative gating behavior. Accordingly, depending on design or selection, multiple input domains can cooperatively regulate the fusion protein in a wide variety of functionalities, including as an OR-gate, an AND-gate, and an AND-NOT-gate. Similarly, a plurality of output domains can be combined in a single fusion protein, to provide more complex switching. Table 3 provides the compositions of exemplary fusion protein switches, including their corresponding output domain, input domains and regulating external ligand. Specification, p.19, lines 3-24.

Those skilled in the art have recognized that the invention is not limited to a single embodiment, but that Applicants' teachings "...pave the way for creating new signal-response elements by protein design. Adler et al. Signaling Breakthroughs of the Year. Adler, Gough, and Ray (2004) Science's STKE 2004: eg1-1. In fact, following the guidance of their disclosure, the inventors have used modular intramolecular interaction domains to engineer novel regulatory control over distinct catalytic output activities. For example, they have demonstrated the ability to apply their method of regulation to Dbl-homology (DH) domains of distinct guanine nucleotide exchange factors (GEFs). They have inserted the engineered GEF switches into cells and used them to precisely alter cell behavior, conferring a new morphological response when stimulated by an appropriate upstream target. They have also demonstrated that they can build these types of autoinhibitory switches that respond to diverse input stimuli. For example, they have engineered switch proteins that are activated by a specific protein kinase by using an autoinhibitory control unit that consists of a PDZ domain-peptide recognition pair whose interaction is disrupted by phosphorylation. They have also built switches with more complex behavior by combining multiple domains. For example, they have built switches that are controlled by 3 inputs simultaneously (by using 3 autoregulatory domain interactions) or switches that show digital type (all-or-none) responses by using multiple autoregulatory interactions of the same type. I am familiar with these types of protein engineering, and in my opinion all these results were predictably obtained following the guidance of their patent application without requiring any undue experimentation.

Accordingly, in my opinion the Specification enables one skilled in the art to make and use without undue experimentation an autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain. The Specification enables one skilled in the art to make and use such fusion proteins with a wide range of alternative output and input domains. Swapping alternative input and output domains in the recited fusion proteins involves only routine gene splicing and activity screening.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: 16 June '06

Henry R Bourne

Henry Bourne, M.D.